

SPECIFICATION

TO ALL WHOM IT MAY CONCERN:

- 5 Be it known that we, TRACEY DOUCETTE, a citizen of Canada, resident of Stanhope, Canada, MELLISA PERRY, a citizen of Canada, resident of Charlottetown, Canada, R. ANDREW TASKER, a citizen of Canada, resident of Charleottetown, Canada, HENRIETTE HUSUM BAK-
JENSEN, a citizen of Denmark, resident of Copenhagen, Denmark and
10 CATHERINE RYAN, a citizen of Canada, resident of Charlottetown, Canada, have invented a new and useful improvement in a

DEVELOPMENTAL ANIMAL MODEL OF TEMPORAL LOBE EPILEPSY

- 15 which invention is fully set forth in the following specification.

DEVELOPMENTAL ANIMAL MODEL OF TEMPORAL LOBE EPILEPSY

FIELD OF INVENTION

The present invention relates to animal models for studying epilepsy and related seizure disorders. More specifically, the invention pertains to a developmental animal model of temporal lobe epilepsy (TLE) that is particularly useful for studying the efficacy of anti-epileptic pharmaceutical compositions. The seizure disorder is produced in non-transgenic animals by treatment in early life with a kainate-receptor agonist, and manifests in a reproducible seizure-like syndrome induced by exposure to mild to moderate stressors.

BACKGROUND OF THE INVENTION

Epilepsy is a family of debilitating neurological disorders that afflicts approximately 4% of the population. The disease often begins in childhood and persists throughout the lifetime of the patient. Although a number of anti-convulsive therapies have been developed for the control of epilepsy, seizures remain uncontrolled in approximately one-third of patients with epilepsy, and treatment failures are common (Loscher, W. and Schmidt, D., 2002, *Epilepsy Res.* 50:3-16).

Accordingly, physicians, scientists and research-based organizations interested in the control and prevention of epilepsy have repeatedly stressed the need for new, clinically correlated animal models with which to study the aetiology of these disorders, and to evaluate potential new therapeutants and therapeutic strategies.

To date, rodent models of epilepsy are restricted to either (a) chemically or electrically - induced acute status epilepticus, (b) spontaneous seizures following chemical or electrical kindling, or (c) genetic models, such as inbred rodent strains that manifest a reduced seizure threshold (Cole, A.J., Koh, S. and Zheng, Y., In: T. Sutula and A. Pikanen (eds.). *Progress in*

Brain Research, 2002, 135:13-23). However, genetic models are also characterized by chronic systemic disease that limits their utility.

For instance, United States Patent No. 5,698,766 discloses a transgenic mouse model lacking 5HT_{2C} receptors. While it is proposed that this animal model is useful for testing drugs against diseases that result in seizures, such as epilepsy, such animals are also overweight due to abnormal feeding behaviour, and are prone to spontaneous death from seizures.

Another transgenic mouse model is described in U.S. Patent Application Publication No. US 2002/0104107 A1, which involves transgenic mice expressing a hypersensitive nicotinic acetylcholine receptor. Although these mice have been proposed as a model system for studying agents that modulate seizures associated with epilepsy, they are primarily useful for examining and manipulating neurobehaviours mediated by nicotine.

Other non-transgenic rodent models have been produced through treatment of the rodent with a neurotoxin. For example, United States Patent No. 5,549,884 discloses a non-transgenic animal produced by inducing brain lesions in the ventral hippocampus of prepubescent rats or mice, by injecting a neurotoxin such as kainic acid into the brain. Once the animal reaches puberty, abnormal behaviour and biological phenomena associated with schizophrenia emerge.

At present, the best animal models available for the study of epilepsy involve acute neurotoxic insults to the mature animal (commonly rats). These models produce motor seizures, changes in cortical EEGs, and neuroanatomical features that approximate human TLE (Ben-Ari, Y. and Cossart, R., *Trend. Neurosci.* [2000] 23:580-587). Epilepsy, however, often appears to have a developmental history beginning in childhood, and is by definition a chronic disease, or at least a disease that manifests itself over a period of time, not simply as a response to an acute insult (Cole, A.J., *et al.* In: T. Sutula and A. Pikanen (eds.) *Progress in Brain Research* [2002] 135:13-23).

Administration of acute high doses of kainic acid, a known kainic acid receptor agonist, has been shown to produce immediate seizures in infant or young rats, but does not cause lasting behavioural, neurochemical, and neuroanatomical correlates of seizure disorders (Stafstrom, C.E., *et al.*, Epilepsia [1993] 34:420-432, Stafstrom, C.E., *et al.*, Brain Res. Dev Brain Res. [1992] 65:227-236, and Sperber, E.F., *et al.*, Brain Res. Dev Brain Res. [1991] 60:88-93). As well, existing chronic models of epilepsy both display and require status epilepticus, causing high mortality, and often underrepresent the complexity of seizure disorders and hence are not always predictive of the clinical profile of many epileptic patients (Loscher, W., Epilepsy Res. [2002] 50:105-123). Thus, existing animal models do not adequately model typical human epilepsy (Cole, A.J., *et al.*, In: T. Sutula and A. Pikanen [eds.] Progress in Brain Research [2002] 135:13-23). Moreover, new animal models that simulate chronic brain dysfunction have been identified as one of the most important prerequisites for advancing epilepsy research (Loscher, W. and Schmidt, D. Epilepsy Res. [2002] 50:3-16).

Therefore, it is recognized that advancing research in epilepsy, and particularly idiopathic epilepsy, requires a developmental animal model; the genesis of which does not rely on an acute traumatic event. The present invention aims to solve this problem by providing a new developmental animal model of temporal lobe epilepsy.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a non-transgenic animal that exhibits a seizure-like disorder, wherein the disorder is induced by subtle manipulation of the glutamatergic system during brain development early in life, and which is useful as a non-human system for studying human seizure disorders such as epilepsy.

Accordingly, one aspect of the invention provides a method of inducing a permanent change in the neurological development of a rodent,

comprising daily treatment of a rodent during the second postnatal week with low doses of a kainate receptor agonist, wherein after treatment with said kainate receptor agonist the rodent exhibits reproducible seizure-like symptoms when exposed to mild to moderate stressors that would not normally elicit a seizure.

The term "second postnatal week" generally refers to the period from about postnatal day (PND) 8 to about PND 14, but includes timeframes slightly outside this precise period, e.g. including a portion of the treatment one or two days outside the PND 8 to PND 14 period.

The kainate-receptor agonist is preferably domoic acid (DOM) or kainic acid, and the daily treatment advantageously involves administration of a single dose of the kainate receptor agonist subcutaneously, each day for a period extending from about PND 8 to about PND 14. Other modes of administering the drug include intravenous, intraperitoneal and intramuscular routes.

The rodents used in this method are preferably neophobic rodents such as rats and mice, with rats being used in especially preferred embodiments.

If domoic acid is used as the kainate receptor agonist, the daily dosage ranges from about 5 to 50 $\mu\text{g/kg}$ of body weight. However, the domoic acid dosage preferably ranges from about 5 to 20 $\mu\text{g/kg}$, and is most preferably about 20 $\mu\text{g/kg}$.

Alternatively, kainic acid may be used in a daily dosage ranging from about 10 to 100 $\mu\text{g/kg}$ of body weight, preferably from about 20 to 50 $\mu\text{g/kg}$, and most preferably about 25 $\mu\text{g/kg}$.

According to another aspect of the invention, there is provided a rodent which has been treated daily with low doses of a kainate receptor agonist during the second postnatal week, resulting in a permanent change in the neurological development of the rodent, wherein the rodent exhibits reproducible seizure-like symptoms when exposed to mild to moderate stressors that would not normally elicit a seizure.

While the seizure-like syndrome is preferably induced by exposure of the rodent to novel environments, such as the Morris Water Maze (MWM), the Novel Water Maze (NWM), or an open field arena, to stress the animal, it could also be induced by other mild to moderate intensity applications of other stressors commonly known to cause seizures when administered intensively, such as chemical convulsants, audiogenic stimuli, or a temperature stress such as hypothermia.

The affected rats may exhibit various physical indicators of a seizure-like syndrome when exposed to stress, although they will typically present with abnormal behaviour characterized by a combination of hunched body posture, facial clonus, mastication with tongue-protrusion, repetitive head extensions and bobbing, repetitive eye blinking/squinting, and vibrissae and ear twitching, often in combination with forelimb clonus.

In adulthood, the treated rats generally exhibit elevated serum oxytocin concentrations and increased expression of hippocampal brain-derived neurotrophic factor (BDNF), with no significant increase in neuropeptide Y (NPY) expression levels..

As a further aspect of the present invention, there is provided the use of a rodent treated according to the method of the present invention, or a rodent of the present invention, for studying the efficacy of a compound or pharmaceutical preparation for treating epilepsy or other seizure-related disorders.

Furthermore, a method of assaying the anti-epileptic efficacy of a compound or pharmaceutical composition is provided by the present invention, wherein the method comprises:

- administering a compound or pharmaceutical composition postulated as having potential as an agent for treating epilepsy or other seizure-related disorders, to a rodent as defined by the present invention,

- exposing the rodent to a form of mild to moderate stress, such as a chemical or environmental stressor, and preferably a novel environment, and
 - measuring the rate of occurrence and/or severity of any seizure induced in the rodent by exposure to the stress,
- wherein a decreased rate of occurrence and/or severity is associated with anti-epileptic efficacy of the compound or pharmaceutical composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a shows the proportion of male rats (n=number of animals) exhibiting Novelty-Induced Seizure-Like (NIS-L) syndrome during Morris Water Maze testing following treatment with 5 or 20 µg/kg domoic acid (DOM) or saline from PND 8 - PND 14 (n=23). Asterisk indicates $p < 0.05$ relative to saline [$X^2(1)=6.54$]. Insert: mean escape latencies (sec) in the Morris Water Maze for male rats. Each block represents mean performance for 4 consecutive trials (4 entry locations) with 2 trial blocks on each test day. Reversal tasks were implemented for Blocks 5 and 9. Error bars indicate standard error. Treatment conditions are: ■ Saline (n=7); ● 5µg/kg DOM (n=8); ▲ 20µg/kg DOM (n=8).

Figure 1b shows the proportion of female rats exhibiting NIS-L during Morris Water Maze testing following treatment with 5 or 20 µg/kg DOM or saline from PND 8 - PND 14 (n=24). Asterisk indicates $p < 0.05$ relative to saline [$X^2(1)=5.34$] and 5 DOM [$X^2(1)=5.34$]. Insert: mean escape latencies (sec) in the Morris Water Maze for female rats. Each block represents mean performance for 4 consecutive trials (4 entry locations) with 2 trial blocks on each test day. Reversal tasks were implemented for Blocks 5 and 9. Error bars indicate standard error. Treatment conditions are: ■ Saline (n=8); ● 5µg/kg DOM (n=8); ▲ 20µg/kg DOM (n=8).

Figure 2a shows the overall proportion of male rats exhibiting NIS-L during at least one of three behavioral tests following treatment with DOM (5 or 20 µg/kg) or saline from PND 8 - PND 14 (n=24). Asterisks indicate

significant differences from saline for both 5 DOM [$X^2(1) = 9.60$, $p < 0.01$] and 20 DOM [$X^2(1) = 16.0$, $p < 0.01$].

Figure 2b represents the overall proportion of female rats exhibiting NIS-L during at least one of three behavioral tests following treatment with DOM (5 or 20 $\mu\text{g/kg}$) or saline from PND 8 - PND 14 ($n=24$). Asterisk indicates significant difference from saline [$X^2(1) = 16.0$, $p < 0.01$] and 5 DOM [$X^2(1) = 9.6$, $p < 0.01$].

Figure 3a shows the expression of BDNF mRNA in either the CA1 or dentate gyrus (DG) hippocampal subfields from 16 month old rats following neonatal administration of either saline (hatched bar; A) or 20 $\mu\text{g/kg}$ DOM (solid bar; B) ($n=7-8$ per group). Asterisk indicates $p < 0.05$ relative to saline control.

Figure 3b shows the expression of NPY mRNA in either the CA1 or dentate gyrus (DG) hippocampal subfields from 16 month old rats following neonatal administration of either saline (hatched bar; A) or 20 $\mu\text{g/kg}$ DOM (solid bar; B) ($n=7-8$ per group). Asterisk indicates $p < 0.05$ relative to saline control.

Figure 4 shows the measured serum oxytocin concentration (pg/ml) in 16 month old rats following neonatal (PND 8 - PND 14) administration of either saline ($n=18$) or 20 $\mu\text{g/kg}$ DOM ($n=16$). Asterisk indicates $p < 0.05$ relative to control.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

Described herein is a developmental animal model for studying temporal lobe epilepsy (TLE) and related seizure disorders. In this animal model, the genesis of an epilepsy-like disorder does not rely on an acute traumatic event, but results from manipulation of the glutamatergic system during brain development in early life. The resulting disorder manifests itself in a reproducible seizure-like syndrome induced by exposure of the animal

to mild to moderate stressors that would not normally induce any form of seizure.

In contrast with other prior art models, which either display seizures spontaneously or require convulsant doses of drugs, animals treated
5 according to the method of the present invention display seizures when exposed only to stimuli that are normally non-convulsive.

According to the invention, the epilepsy-like disorder is induced in a rodent by administering low doses of a kainate-receptor agonist during early brain development. The actual timeframe wherein this early brain
10 development occurs will vary depending upon the classification of the animal. Accordingly, the desired effect may be obtained by treating the rodent prenatally or early in life. However, the inventors have found that the treatment preferably occurs early in life, essentially during the second postnatal week. This treatment regime most advantageously extends from
15 about postnatal day (PND) 8 to PND 14.

The rodents used in this method are preferably rats, although it is envisioned that by adjustment of the drug dosage and administration period to account for inter-species differences in development, similar effects may be obtained in other rodents as well. Particularly advantageous species of
20 rodents are those that are known to be neophobic (ie. avoid novelty), such as rats and mice.

While the kainate-receptor agonist may be administered in one or more daily doses, it has been found that subcutaneous administration of the drug in a single daily dose is particularly advantageous. The drug may also
25 be administered via different routes, such as intravenous, intraperitoneal and intramuscular routes, although different drug dosages may be required.

Domoic acid, a known kainate receptor agonist (Verdoorn, T.A., *et al.*, Eur. J. Pharmacol. [1994] 269:43-49), is a preferred treatment agent for use in the present method. It is used in a daily dosage ranging from about 5
30 to 50 µg/kg. The domoic acid dosage preferably ranges from about 5 to 20 µg/kg, and is most preferably about 20 µg/kg.

Alternatively, kainic acid may be used as the kainic acid receptor agonist. However, due to its comparatively lower potency, kainic acid must be used in higher dosages than are typically required for domoic acid. Accordingly, the daily dosage of kainic acid ranges from about 10 to 100
5 $\mu\text{g/kg}$. The preferred kainic acid dosage ranges from about 20 to 50 $\mu\text{g/kg}$, and is most preferably about 25 $\mu\text{g/kg}$.

While domoic acid and kainic acid are the only known kainate receptor "selective" agonists at present, it is to be understood that any kainate receptor selective agonist may be used to elicit the seizure-like
10 syndrome described herein.

Rodents treated according to the above-described method exhibit a seizure-like syndrome when exposed to mild to moderate stressors, particularly when placed in a novel physical environment such as the Morris Water Maze (MWM), the Novel Water Maze (NWM), or an open field arena.
15 However, stress factors other than novel environments, including low doses of chemical convulsants, auditory stimuli and temperature stress have also been shown to induce a seizure-like response. The "temperature stress" is typically induced in the animal by applying low temperatures, causing hypothermia.

20 Symptoms of the seizure-like syndrome are generally characterized as a combination of abnormal behaviours, including hunched body posture, facial clonus, mastication with tongue-protrusion, repetitive head extensions and bobbing, repetitive eye blinking/squinting, and vibrissae and ear twitching, often in combination with forelimb clonus. Herein, when a rodent
25 presents of all of the above behaviours in response to being placed in a novel environment, the seizure-like syndrome is referred to as "novelty-induced seizure-like (NIS-L) syndrome". In separate embodiments, physical indicators of a seizure-like response other than those described above may be observed when an animal treated according to the present method is
30 exposed to mild to moderate stressors. These physical indicators, which will be recognized by a person skilled in the art, should be considered as

seizure-like symptoms characteristic to an animal treated according to the method of the present invention.

In domoic acid-treated animals exhibiting NIS-L syndrome, increased hippocampal brain-derived neurotrophic factor (BDNF) expression levels are frequently observed, with no significant changes in neuropeptide Y (NPY) expression.

BDNF has been suggested to be involved in epileptogenesis, and both pro- and anti-epileptic effects have been reported, although precise relationships are unclear (Binder, D.K., *et al.*, *Trend Neurosci.* [2001] 24:47-53). BDNF rapidly enhances synaptic transmission among hippocampal neurons, and increases in BDNF appear to correlate with epilepsy in both clinical populations (Takahashi, M. *et al.*, *Brain Res.* [1999] 818:579-582) and rodent models of TLE (Gall, C.M., *Exp. Neurol.* [1993] 124:150-166). However, in most existing rodent models of TLE, a corresponding increase in NPY biosynthesis is also present (Vezzani, A. *et al.*, *Neuroscience* [1999] 90:1445-1461), and has been reported to be neuroprotective and anti-convulsive (El Bahh, B., *et al.*, *Brain Res.* [2001] 894:209-217). In contrast, Takahashi *et al.* report no increase in NPY concentrations in human patients with epilepsy (Takahashi, M., *et al.*, *Brain Res.* [1999] 818:579-582).

Accordingly, the increase in hippocampal BDNF gene transcription with no significant increase in NPY observed in rodents treated according to the method of the present invention corresponds well with reported human data. Moreover, it is notable that BDNF expression and release has been proposed to be regulated by non-NMDA receptors (Wetmore, C., *et al.*, *J. Neurosci.* [1994] 14:1688-1700), since in the current model the treatment paradigm targets non-NMDA receptors (ie. kainic acid receptors) during critical periods of brain maturation.

Marked disturbances in physiological homeostasis occur in epilepsy, including alterations in neuroendocrine function (Cole, A.J., *et al.*, In: T. Sutula and A. Pikanen (eds.) *Progress in Brain Research* [2002] 135:13-23). The paraventricular nucleus (PVN) of the hypothalamus plays a significant

role in neuronal responses to stressful stimuli (Jezova, D., et al., Ann. N. Y. Acad. Sci. [1995] 771:192-203) and it has been shown that generalized seizures cause an activation of oxytocin containing neurons in the PVN with long-lasting (minimum 8 weeks) increases in mRNA (Sun, Q., *et al.*, Neuroscience [1996] 71:543-554, and Ben-Ari, Y. and Cossart, R., Trend. Neurosci. [2000] 23:580-587). According to the present invention, adult rodents treated neonatally with low-levels of a kainic acid receptor agonist show elevated serum oxytocin concentrations, which further strengthens the idea that the behavioural manifestations that define NIS-L are indeed correlates of clinical epilepsy.

In addition to progressive onset, it is known that in the human population, stress is a common seizure-provoking factor for patients with temporal lobe epilepsy (Foldvary, N. and Wyllie, E. Epilepsy. In: C.G. Goetz (Ed) Textbook of Clinical Neurology 2nd ed. [2003] Elsevier Science, New York, pp. 1155-1187). Thus, it is significant that novel environments that present as mild to moderate stressors reliably and reproducibly induce the NIS-L syndrome.

Although not confirmed, it is probable that the NIS-L syndrome arises from altered glutamatergic function. The glutamate system in early developmental stages can be altered by many mechanisms (Ikonomidou, C. et al., Science [2000] 287:1056-1060, and Johnston, M.V., et al., In: B.S. Meldrum, F. Moroni, R.P. Simon, and J.H. Woods (Eds.) Excitatory amino acids. [1991] Raven Press, New York pp. 711-715), and critical aspects of brain development are activity-dependent. One hypothesis, therefore, is that early manipulation of the glutamate system may stabilize immature synaptic connections normally destined for removal, thereby resulting in an intrinsically hyperexcitable brain in adulthood. Thus, in this new model of epilepsy, the onset is triggered early in development by subtly altering glutamatergic tone, and in particular, by targeting kainic acid receptors. Abnormal kainic acid receptor expression has been noted in hippocampal tissue from human patients with epilepsy (Grigorenko, F. *et al.*, J. Neurol.

Sci. [1997] 9:35-45), has been reported in animal models of temporal lobe epilepsy (Telfeian, A. E., *et al.*, Neurobiol. Dis. [2000] 7:362-374), and is subject to developmentally-regulated post-translational editing that controls excitability (Bernard, A. *et al.*, Eur. J. Neurosci. [1999] 11:604-616).

- 5 Therefore, early kainic acid receptor activation may cause a permanent alteration in kainic acid receptor subunit expression, resulting in a mature organism with circuitry that is hyperexcitable.

Accordingly, the animal model described herein provides a useful non-human system for studying temporal lobe epilepsy and other seizure-related disorders, and is especially useful as a tool for studying the effect of compounds and pharmaceutical preparations for treating epilepsy.

Methods of testing the efficacy of compounds or pharmaceutical compositions in animals are known in the prior art, and may be easily adapted for assaying anti-epileptic efficacy using rodents of the present invention. While other embodiments may be envisioned, it will be expected that these testing methods will generally include steps of; administering a compound or pharmaceutical composition thought to have potential as an agent for treating epilepsy or other seizure-related disorders to a rodent defined by the present invention; exposing the rodent to a novel environment or other form of mild to moderate stress; and measuring the rate of occurrence and/or severity of any seizure induced in the rodent by exposure to the mild to moderate stress, wherein a decreased rate of occurrence and/or severity is associated with anti-epileptic efficacy of the compounds or pharmaceutical composition.

25 All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications, patents and patent applications are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

The invention will be further understood from the following non-limiting examples. The following examples are provided to describe in detail some of the representative, presently preferred methods and materials of the invention. These examples are provided for purposes of illustration of the inventive concepts, and are not intended to limit the scope of the invention as defined by the appended claims.

EXAMPLES

Example 1:

Within 24 hr of birth, offspring of untimed-pregnant Sprague Dawley rats (Charles River Laboratories, St. Constant, PQ) were culled to 10 pups/litter (5 males and 5 females). From post-natal day (PND) 8 - PND 14, pups were weighed and given a single daily injection (s.c.; 10 ml/kg) of either saline, or 5 or 20 µg/kg domoic acid (DOM). These doses are well below those normally required to induce toxicity in animals of this age (Doucette, T.A., *et al.*, Neurotox. Teratol. [2003] 25:473-479, and Doucette, T.A., *et al.*, Neurotox. Teratol. [2000] 22:863-869). Pups were assessed daily until weaning (on PND 22) for overt signs of toxicity, and all pre- and post-weaning testing was conducted with the experimenter blind to treatment. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care. None of the pups displayed any signs of physical or behavioural toxicity prior to weaning and there were no significant differences in weight gain between groups. Rats received no additional drug treatment after PND 14. On PND 120, animals were tested for spatial learning and memory in the Morris Water Maze (MWM) (8 trials/day over 6 consecutive days), using a standard reversal procedure (McNamara, R.K. and Skelton, R.W., Brain Res. Brain Res. Rev. [1993] 18:33-49). While on the escape platform during the 60 second inter-trial interval, a significant number of DOM-treated rats presented with abnormal behaviour characterized by a combination of hunched body posture, facial clonus, mastication with tongue-protrusion, repetitive head extensions and

bobbing, repetitive eye blinking/squinting, and vibrissae and ear twitching, often in combination with forelimb clonus. Presentation of all of the above behaviours was termed “novelty-induced seizure-like (NIS-L) syndrome”. Of note, the expression of this syndrome was not consequent to earlier drug-induced toxicity or seizures, nor was it spontaneous. Analyses revealed that a significant proportion of DOM-treated rats exhibited the NIS-L syndrome ($X^2(2) = 6.46$, $p < 0.05$ for males and $X^2(2) = 9.63$, $p < 0.01$ for females), with no effect on standard measures of MWM performance and no false positives in control animals (Fig. 1a and b).

To determine if the syndrome could be elicited by novel stimuli other than the MWM, all rats were tested in the Novel Water Maze (NWM) (Kant, G.J., Pharmacol. Biochem. Behav. [1993] 44:287-295) approximately 120 days after the MWM testing (i.e. PND 240). Again, a significantly greater number of drug-treated animals exhibited the NIS-L syndrome compared to saline-treated animals in the NWM for both male, ($X^2(2) = 6.0$, $p < 0.05$ [saline: 0%; 5 DOM: 33.3%; 20 DOM: 66.7%]), and female ($X^2(2) = 8.66$, $p < 0.05$ [saline: 0%; 5 DOM: 33.3%; 20 DOM: 100%]) rats. To determine the response to a novel terrestrial environment, thirty days later (i.e. PND 270) male rats were tested in an open field arena and also displayed NIS-L, ($X^2(2) = 6.00$, $p < 0.05$ [saline: 0%; 5 DOM: 50 %; 20 DOM: 66.7%]). Even higher proportions of treated animals exhibited NIS-L during at least one of the three behavioural tests ($X^2(2) = 17.83$, $p < 0.01$ for male and $X^2(2) = 17.83$, $p < 0.01$ for female rats) (Fig. 2).

Example 2:

Current animal models of epilepsy present with various neurochemical correlates. To determine whether the NIS-L syndrome was characterized by such changes, serum samples and brain tissue from a separate cohort of animals treated neonatally (PND 8 - PND 14) with saline or 20 $\mu\text{g/kg}$ DOM were collected. In this experiment, 50% of both male and female drug-treated animals displayed the NIS-L syndrome during NWM

testing (males $X^2(1) = 5.89$, $p < 0.05$ and females $X^2(1) = 5.89$, $p < 0.05$) at 15 months, while no animals neonatally treated with saline displayed evidence of this syndrome during similar NWM testing.

One month following NWM testing (PND 480), brain tissue obtained following rapid dissection and freezing in liquid nitrogen was assayed for hippocampal brain-derived neurotrophic factor (BDNF) and neuropeptide Y (NPY) mRNA expression using *in situ* hybridization. DOM-treated rats showed a significant elevation in BDNF gene expression in the CA1 region ($F(1,10) = 7.88$, $p < 0.05$) relative to saline controls (Fig. 3a). Similarly, elevated BDNF gene expression in the dentate gyrus approached significance for DOM-treated rats relative to saline counterparts, $F(1,10) = 4.74$, $p = 0.055$ (Fig. 3a). No group differences, however, were found for hippocampal NPY mRNA expression in either the CA1 region (Fig. 3b) or the dentate gyrus (Fig. 3b).

Serum samples were assayed for circulating oxytocin levels using radioimmunoassay (RIA). A significant elevation in circulating oxytocin was found in DOM-treated rats relative to saline controls ($F(1,28) = 15.41$, $p < 0.05$) (Fig. 4), and further, a significant correlation was found between the expression of NIS-L and oxytocin levels ($r(30) = 0.341$, $p < 0.05$).

20

Example 3:

To determine whether the syndrome was specific to DOM, or could be elicited by other kainate- receptor agonists, a third study was conducted, parallel in design to Example 1, but using equi-efficacious doses of kainic acid (KA) (saline, 25 and 100 $\mu\text{g/kg}$ KA) (Doucette, T.A., *et al.*, Neurotox. Teratol. [2000] 22:863-869). Similar to DOM-treated rats, the NIS-L syndrome occurred only in those rats treated neonatally with drug (male, $X^2(2) = 8.58$, $p < 0.05$ [saline: 0%; 25 KA: 62.5%; 100 KA: 62.5%] and female, $X^2(2) = 6.42$, $p < 0.05$ [saline: 0%; 25 KA: 16.7%; 100 KA: 57.1%] rats), with no differences present in MWM performance (data not shown). In addition, KA-treated male rats tested in the open field arena exhibited the NIS-L

30

syndrome ($X^2(2) = 7.19$, $p < 0.05$, saline: 0%; 25 KA: 62.5%; 100 KA: 33.3%). As with Example 1, no false-positives for NIS-L in saline-treated animals were noted in any test, confirming that drug treatment during early development was responsible for the NIS-L syndrome.

5

Behaviour Testing:

The Morris Water Maze (MWM) consisted of a circular tank measuring 1.5 m in diameter. Water temperature was maintained at approximately 21°C and was made opaque by a layer of polypropylene pellets floated on the surface. Four points around the edge of the pool were arbitrarily designated as north (N), south (S), east (E), and west (W), allowing the apparatus to be divided into 4 corresponding quadrants (NE, SE, NW, SW). A clear plexiglass escape platform was submerged approximately 2 cm below the water surface and placed, initially, in the NE quadrant of the maze (alternate locations for the reversal task were implemented on Day 3 and Day 5). Extra-maze cues consisted of laboratory furniture and lights (held constant throughout the experiment). A video camera was mounted above the center of the pool and all performance was recorded for subsequent analyses. Rats were given 8 trials/day (administered as two consecutive blocks of 4 trials, with start locations pseudo-randomly selected without replacement within each block of 4 trials) for each of 6 test days (60 sec trial, 60 sec inter-trial interval during which time the rat remained on the escape platform). If the rat did not find the escape platform within the allotted time it was guided to the finish by the experimenter. A 60 sec probe trial was administered 24 h following the last test day. Escape latencies and inter-trial behaviour was recorded by observers blind to experimental treatment.

The Novel Water Maze (NWM) (Kant, G.J., Pharmacol. Biochem. Behav. [1993] 44:287-295) was selected based on its provision of a novel environment within which to evaluate the novelty impact on the precipitation of NIS-L. Animals were tested for 6 consecutive swim trials of 60 sec

duration and scored for the presence or absence of NIS-L behaviours, by an observer blind to treatment during the 120 sec inter-trial period.

The open field apparatus consisted of a 1.5 m diameter circular arena sectioned into 32 grids (16 central grids: 9 cm x 9 cm; 16 peripheral grids).

- 5 The open field was chosen as another environment that provided novelty while at the same time being less physiologically challenging than the previously described two water maze tasks. All rats were given a single 10 min trial in the open field arena and scored for the presence or absence of NIS-L by an experimenter blind to treatment. All testing procedures were
10 conducted according to the guidelines of the Canadian Council on Animal Care.

***In situ* Hybridization:**

- Coronal brain sections (15 µm) at the level of the dorsal hippocampus
15 (-3.14 to -4.16 mm relative to bregma) (Paxinos, G. and Watson, C., The rat brain in stereotaxic coordinates [1986] Academic Press, Inc.) were cut on a cryomicrotome and thaw-mounted onto Superfrost™ glass slides with four consecutive coronal sections per slide. The slices were briefly dried on a hot plate and then transferred to a -80°C freezer until further use. For *in situ*
20 hybridization, slides were defrosted, left for 10 min at room temperature (RT) to evaporate condensed water and subsequently placed for 5 min in 4% formaldehyde in PBS (0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄), rinsed briefly and then placed for 5 min in PBS. Subsequently, the sections were placed in 70% EtOH for 5 min and finally placed for an additional 5 min
25 in 95% EtOH. Synthetic oligonucleotide DNA probes (DNA technology ApS, Aarhus, Denmark) were used for the visualization of preproNPY mRNA (5'GTCC-TCTG-CTGG-CGCG-TCCT-CGCC-CGGA-TTGT-CCGG-CTTG-GAGG-GGTA3', disclosed in Larsen, P.J., *et al.*, J. Neurosci. [1993] 13:1138-1147) and BDNF mRNA (5'GGTC-TCGT-AGAA-ATAT-TGGT-TCAG-TTGG-CCTT-TTGA-TACC-GGGAC3', disclosed in Zetterstrom, T.S.,
30 *et al.*, Brain Res. Mol. Brain Res. [1998] 57:106-110). Each probe was 3'tail

labelled with [$\alpha^{35}\text{S}$]dATP (>3000 Ci/mmol, Amersham) using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Denmark). The labelled probe was added to give a specific activity of 3.0×10^5 cpm/100 μL to the hybridization buffer containing 50% formamide (v/v), 4x saline-sodium-citrate (SSC) (1x SSC = 0.15 M NaCl, 0.015 M $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ pH 7.0), 10% (w/v) dextran sulfate, and 10 mM dithiotreitol. 100 μL of hybridization mixture was added onto each slide while placed in a humidity box. The slides were cover-slipped and left at 42°C overnight. The slides were subsequently washed for 60 min in 1x SSC at 60°C, passed through a series of 1 min rinses in 1x SSC, 0.1x SSC, 70% EtOH and 95% EtOH at RT, and finally air-dried. Hyperfilm β -max were exposed to the slides for 3 weeks with a ^{14}C -microscale (both Amersham, Denmark) and then developed in a Kodak D19 film developer. Computer-assisted image analysis was performed using the Scion ImageTM computer analysis program (NIH, USA) for quantification of hybridization signals and ^{125}I -PYY binding. For the quantification of BDNF and NPY mRNA levels, optical densities were measured bilaterally in four adjacent sections per animal, over the dentate gyrus (the granular and hilar area) and CA1 area (strata oriens and radiatum) of the hippocampus. Values were averaged per section and subsequently per animal. Absolute values expressed as pCi per mg are representing the total mean of the group.

Radioimmunoassay:

Approximately 3.0 ml of trunk blood was collected from each animal in 1.5 ml microcentrifuge tubes, left to cool, clot and coagulate for 3 min, and then centrifuged at 16,000x g for 10 min. Serum was placed in cryovials and stored at -40°C until further use. Serum oxytocin was measured in duplicate by commercial radioimmunoassay (Phoenix Pharmaceuticals Inc., Belmont, CA). This kit required 2 overnight incubations ($K_{50}=5.89$ pg/tube with 100% cross-reactivity with oxytocin). All reagents were reconstituted with the RIA buffer. Total counts (TC) tubes, non-specific binding (NSB)

tubes, total bound (TB) tubes, standards and samples tubes were labelled in duplicate. 200 µl of RIA buffer was pipetted into each NSB tube and 100 µl of primary antibody (rabbit anti-oxytocin serum) was pipetted into the TB tubes, standards and samples. The contents of each tube were vortex
5 mixed and incubated overnight at 4°C. 100µl of ¹²⁵I-oxytocin tracer solution was added to each tube. The contents of each tube were then vortex mixed and again incubated overnight at 4°C. 100µl of goat anti-rabbit IgG serum and normal rabbit serum were added to each tube except the TC tubes. The tubes were incubated at room temperature for 90 min. Following this
10 incubation, 500µl of RIA buffer was added to each tube except the TC tubes. All of the tubes were centrifuged at 1,700x g for 20 min at 4°C. Supernatant was aspirated immediately following centrifugation except for TC tubes and pelleted residue was placed in a gamma counter (Riastar, Canberra Packard, Canada). Average values for duplicate samples were
15 expressed as pg oxytocin / ml.